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Penicillium stoloniferum Virus: Altered Replication in Ultraviolet-derived Mutants

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SUMMARY

Phenotypic mutants of the wild type of *Penicillium stoloniferum* NRRL 5267 were obtained from conidia exposed to ultraviolet light for 60 min (10% survival). Virus content of the wild type and of nine phenotypic mutants was determined by polyacrylamide gel electrophoresis. Four mutants had no detectable *Penicillium stoloniferum* virus F (PsV-F), whereas the other five had levels of PsV-F in the mycelium similar to the wild-type strain. All nine mutants and the wild type had comparable levels of *Penicillium stoloniferum* virus S (PsV-S). Maximum virus levels occurred after 9 days of submerged culture in a 2% yeast extract-15% sucrose medium. Virus replication in the fungal host continued after protein, RNA and DNA synthesis levelled off. Virus levels ranged from 85 to 150 E_{260} units (extinction units at 260 nm in 1 cm cell) per 4·7 to 5·3 g dry weight of mycelium for the mutant strains compared to 106 E_{260} units per 4·2 g dry weight of the wild-type strain.

INTRODUCTION

Ellis & Kleinschmidt (1967) presented the first evidence that *Penicillium stoloniferum* contained a polyhedral virus. Banks *et al.* (1968) subsequently isolated virus particles that contained double-stranded RNA (dsRNA). Two electrophoretically and serologically distinct viruses PsV-F and PsV-S have been isolated from *P. stoloniferum* NRRL 5267 (Buck & Kempson-Jones, 1970, 1973; Bozarth, Wood & Mandelbrot, 1971). The virus with the highest electrophoretic mobility (PsV-F) contains three distinct mol. wt. classes of dsRNA (0·99, 0·89 and $0{\cdot}23 \times 10^6$) whereas the PsV-S contains two dsRNA classes (1·1 and $0{\cdot}94 \times 10^6$) (Bozarth *et al.* 1971). Infection of virus-free isolates of *P. stoloniferum* with PsV-F and PsV-S during heterokaryon formation and infection of protoplasts by PsV-S has been reported (Lhoas, 1971*a*, *b*).

Detroy, Freer & Fennell (1973) have observed a correlation between the production of mycophenolic acid, an antiviral fungal metabolite, and the absence of virus in *Penicillium stoloniferum*. Several strains of *P. stoloniferum* that produce mycophenolic acid contained no detectable virus; the virus-containing isolate produced no detectable mycophenolic acid.

Although pertinent evidence now exists for infection and production of mycoviruses, limited information exists on *in vivo* virus replication. We have studied the *in vivo* replication of PsV-F in *Penicillium stoloniferum* NRRL 5267 and compared maximum virus levels in phenotypic colour mutants derived from *P. stoloniferum* NRRL 5267 as donor strains in heterokaryon–virus transfer studies.

METHODS

Organisms and growth conditions. The virus-containing strain of *Penicillium stoloniferum* Thom NRRL5267 (ATCC14586) was supplied by the ARS Culture Collection maintained at the Northern Regional Research Laboratory. The organism was routinely maintained on PDA (potato dextrose agar) slants. To produce phenotypic colour mutants, spores of *P. stoloniferum* NRRL 5267 were washed from PDA slants with 0·01% Triton X and exposed to long and shortwave u.v. radiation in a Chromato-vue Model C-3 up to 60 min (10% survival). Nine colonies having different phenotypic colour characteristics were selected for further study. The mutants were maintained on PDA. Conidia (10^6 to 10^7) in 0·01% Triton X were used to inoculate each 500 ml Erlenmeyer flask containing 100 ml 2% yeast extract and 15% sucrose (YES) medium. All isolates were grown at 28 °C on a Brunswick rotary shaker at 250 rev/min.

To determine the rate of viral dsRNA replication in the wild-type *Penicillium stoloniferum* NRRL 5267, flasks were harvested at various intervals from 24 to 155 h. Mycelia for comparison of mutant and wild-type strains were harvested at 2-day intervals from 5 to 11 days' growth, since preliminary results had indicated that maximum virus levels occurred during this period.

Estimation of virus by viral dsRNA. For the study of replication of the virus in the wild-type strain, mycelia were suspended in 0·1 M-PO_4 buffer, pH 7·2 (50 ml buffer/g wet weight mycelium) and transferred to 75 ml glass Bronwill mechanical cell homogenizer flasks containing 45 g of 1 mm glass beads. Each sample was homogenized for 3 min at 4000 rev/min under a cold CO_2 stream at 0 to 1 °C. The homogenates were centrifuged at 8000 ***g*** for 10 min to remove cell debris and the supernatant fluid (SNF) then centrifuged at 105000 ***g*** for 2·5 h. The pellet was resuspended in 2 ml of PO_4 buffer and subjected to low-speed centrifuging at 8000 ***g***. The SNF was mixed with an equal vol. of aqueous 90% phenol containing 0·1% (w/w) 8-hydroxy quinoline (Cox, Kanagalingam & Sutherland, 1970), shaken for 20 min at 25 °C and centrifuged at 4000 ***g*** for 20 min. Nucleic acid was freed from phenol by repeated precipitation from 0·2 M-sodium acetate with an equal vol. of cold methanol. The precipitate was dissolved in a minimum vol. of saline citrate (SSC) solution (0·15 M-NaCl + 0·015 M-sodium citrate), pH 7·4.

The RNA samples were incubated with 1·0 μg/ml ribonuclease B (Sigma Chemical Co.) in 0·3 M-saline-tris-EDTA (STE) buffer (0·3 M-NaCl, 0·01 M-tris and 0·001 M-EDTA) at 37 °C for 30 min. The remaining RNA was precipitated with cold methanol, the mixture centrifuged at 8000 ***g*** for 10 min, the pellet redissolved in SSC buffer and the final solution subjected to polyacrylamide gel electrophoresis. Mobilities of RNA components were compared to a pure, *Penicillium stoloniferum* dsRNA standard. The dsRNA was measured by integration of the areas under the electrophoretic peaks compared to a dsRNA standard.

PsV-F content in wild-type and mutant strains. Mycelia (15 g wet weight, in 75 ml of PO_4 buffer at 2 °C) were ruptured by passage through a Manton-Gaulin homogenizer at 6000 lb/in^2 pressure. The homogenates were centrifuged at 16000 ***g*** for 20 min to remove cellular debris. Polyethylene glycol (PEG) 6000 6% (w/v) and NaCl 2·8% (w/v) was added to 40 ml of the SNF, and the mixture was stored for 1 h at 4 °C. The precipitate was collected by centrifuging at 12000 ***g*** for 10 min. The virus pellets were resuspended in 2 ml each of 0·1 M-PO_4 buffer, and debris was again removed by low-speed centrifuging at 12000 ***g*** for 10 min. The supernatant fluid was stored at −20 °C before gel electrophoresis.

Measurement of PsV-F. Polyacrylamide gels (2·4% acrylamide, 0·15% bisacrylamide and 0·5% agarose in TAE–SDS buffer (0·09 M-tris; 0·07 M-sodium acetate; 0·002 M-EDTA and

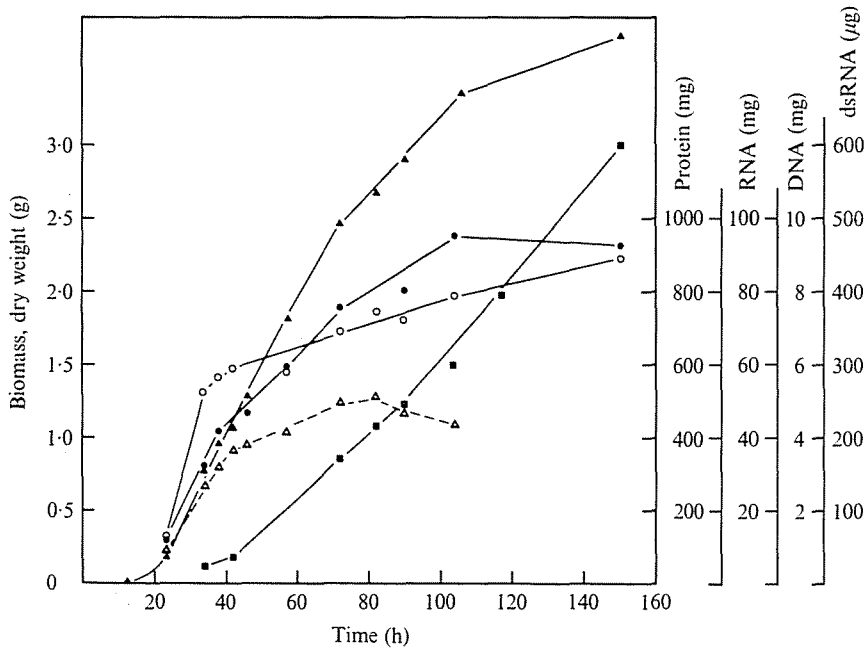

Fig. 1. *In vivo* virus dsRNA replication and host DNA, RNA and protein synthesis. ▲—▲, dry weight; ●—●, protein; ○—○, RNA; △—△, DNA; ■—■, dsRNA.

0·5% (w/w) SDS, pH 7) were prepared essentially as described by Loening (1967). Electrophoresis was carried out for 3 h at 6·5 mA per tube at 25 °C. Gels were scanned at 260 nm by a Gilford linear transport system. A PsV-F standard with 10 E_{260} units (extinction units at 260 nm in 1 cm cell) per ml was prepared by sucrose gradient sedimentation and analysed by gel electrophoresis for purity. A PsV-F standard curve was determined since the area under the PsV-F peaks showed a linear relationship to the amount of PsV-F applied between 0·03 and 0·25 E_{260} units of standard PsV-F.

Qualitative detection of PsV-S. Because PsV-S occurs in *Penicillium stoloniferum* tissue at one-tenth the level of PsV-F and is considerably less stable than PsV-F, no attempt was made to determine PsV-S levels in E_{260} units, However, its presence was qualitatively monitored by comparison to a standard preparation containing both PsV-F and PsV-S.

Measurement of total RNA, DNA and protein in mycelial homogenates. The SNF from mycelial homogenates was assayed for RNA by the orcinol method of Brown (1946), for DNA by the method of Burton (1956) and for protein by the method of Lowry *et al.* (1951).

Mycophenolic acid detection. Mycophenolic acid was detected according to the method of Detroy *et al.* (1973) which uses $FeCl_3$ to visualize mycophenolic acid following thin-layer chromatography on silica gel plates.

RESULTS

Virus replication in NRRL5267 *wild-type strain*

In vivo virus replication was examined from 20 to 155 h by using the levels of dsRNA to measure the combined amount of PsV-F and PsV-S present. Viral dsRNA levels and host DNA, RNA and protein levels are depicted in Fig. 1. Germination in the YES medium

Table 1. *Maximum levels of virus (PsV-F) found in the mycelium of* Penicillium stoloniferum NRRL5267 *and u.v.-derived phenotypic colour mutants*

Isolate	Spore colour	Total dry wt. flask (g)*	Total PsV-F in E_{260} units* flask
NRRL5267	Green	4·2	106
Group A			
1	White	5·0	87
2	Grey-green	4·8	139
3	Grey	4·7	98
4	White	4·9	150
5	Grey	5·3	85
Group B			
6	Tan	4·6	ND†
7	Tan	4·7	ND
8	Tan	4·8	ND
9	Tan	5·0	ND

* Average of total PsV-F and total dry weight of mycelia harvested from two flasks containing 100 ml yeast extract-sucrose medium. Maximum levels of virus and total dry weight are for 9 days' fermentations, except for mutants 1 and 2 in which maximum PsV-F levels were observed at 11 days.

† Not detectable. The assay was capable of detecting one E_{260} unit/flask.

began at 16 h, followed by rapid macromolecular synthesis. The viral dsRNA was first detected at 30 h, followed by a steady increase in dsRNA levels through 155 h. The increase of dsRNA continued after DNA, RNA and protein had reached near maximum levels.

Level of PsV-F in wild-type and phenotypic mutant strains

Direct measurement of the PsV-F peaks on acrylamide gels was used to compare wild-type and mutant strains between 5 and 11 days. Total PsV-F content increased in the wild-type strain through 9 days of growth, followed by a decline after 10 days of growth. The respective E_{260} units of PsV-F/g dry weight of mycelia were 9·2, 15·6, 25·2 and 18 for 5, 7, 9 and 11 days of growth, respectively.

Analysis of the PsV-F content of the u.v.-derived mutants revealed two specific groups. Five of the mutants had maximum PsV-F levels comparable to the wild-type strain (group A) and four other mutants had no detectable PsV-F particles (group B) (Table 1, Fig. 2).

Group A and B mutants contained PsV-S (Fig. 2). The level of PsV-S in all isolates was comparable to the wild type.

The dsRNA isolated from the virus preparations of group B mutants was resistant to pancreatic ribonuclease at 1 × STE, but susceptible to hydrolysis at 0·1 × STE. The electrophoretic mobility of the dsRNA was similar to the 0·94 and $1{\cdot}1 \times 10^6$ mol. wt. classes of viral RNA reported for PsV-S dsRNA (Bozarth *et al.* 1971).

There were no significant differences in the biomass yields between the two mutant groups or wild-type strain between 5 and 11 days of growth. Maximum yields of PsV-F for group A mutants ranged from 85 to 150 E_{260} units per flask. Maximum levels represent a two to sixfold increase over levels found at 5 days of growth in the various isolates. No PsV-F was found in the spent media through 11 days of culture.

No mycophenolic acid was detected in the culture filtrates of *Penicillium stoloniferum* NRRL5267 or the nine mutants derived from it.

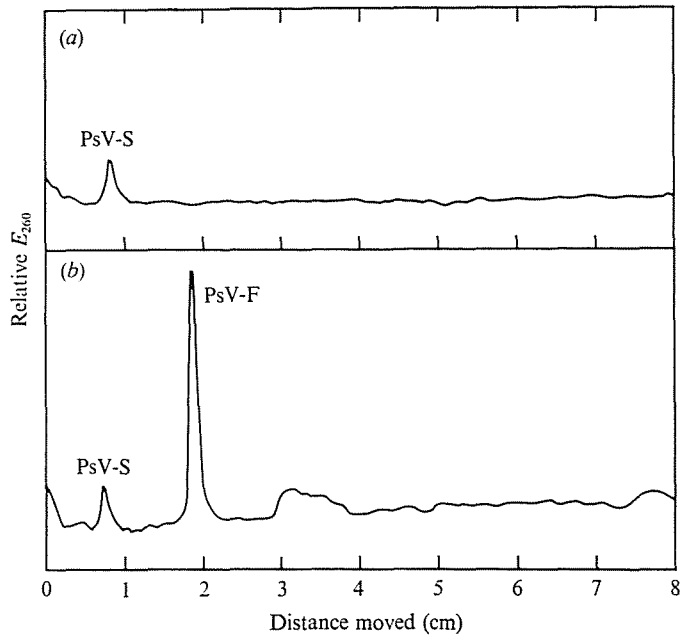

Fig. 2. Electrophoretogram of acrylamide gels following electrophoresis of a typical virus preparation of (*a*) group B mutants with only PsV-S; (*b*) wild-type *Penicillium stoloniferum* NRRL5267 or group A mutants with both PsV-F and PsV-S. The 2·4% gels were run at 6·5 mA per tube for 3 h.

DISCUSSION

After the initial rate of protein, RNA and DNA synthesis declines, viral dsRNA and PsV-F replication continues. Nucleic acid turnover after decline in primary macromolecular synthesis could lead to a build-up of nucleotide pool levels and continued virus dsRNA synthesis and virus development. PsV-F levels in the wild type increased fourfold between 5 and 9 days while biomass increased 1·4 times. This continued synthesis of PsV-F would demand a readily available nucleotide pool.

The absence of virus particles in spent concentrated fermentation broths up to 11 days demonstrates that no lytic process is initiated even at high virus titre. Other workers (Banks *et al.* 1968; Detroy, Lillehoj & Hesseltine, 1974) investigating the *Penicillium stoloniferum* virus system have reported the release of virus from mycelia in a complex corn steep-liquor medium. Apparently virus release was not virus-mediated but was probably the result of general autolysis of the mycelia. This increased stability of the fungus in YES compared to corn steep-liquor medium allows for accumulation of high levels of virus in the fungal host.

Two mechanisms could explain the loss of PsV-F replication in the group B mutants. One possibility is the direct degradation or dissociation of the structural integrity of the virus genome by u.v. irradiation with a loss of virus assembly or replication. This mechanism, however, does not account for the apparent lack of effect of u.v. treatment on virus replication in the group A mutants reported here or the survival of virus in *Penicillium chrysogenum* strains derived by u.v. irradiation reported by Banks *et al.* (1969). Acquisition of host resistance to the virus through a mutation in the fungal genome is a second possible mechanism to explain the loss of PsV-F replication. Genetically determined host control of virus replication exists for many plant viruses (Matthews, 1970).

A recent report of the possible involvement of virus-like particles with the killer character

in the yeast *Saccharomyces cerevisiae* provides an example of a fungal system where host resistance to virus infection is under nuclear control. In this system two classes of dsRNA called P1 and P2 are believed to be separately encapsidated (Herring & Bevan, 1974). The maintenance of P2 dsRNA is dependent on the dominant M nuclear gene and is lost from the host that has the recessive m gene (Bevan, Herring & Mitchell, 1973). The m gene imparts host resistance to the virus-like particles with P2 dsRNA but not to virus-like particles with P1 dsRNA. Replication of the mycovirus containing P1 dsRNA is independent of the m allele.

We postulate that a genetic mechanism may also control resistance to virus replication in *Penicillium stoloniferum*. A mutation in a susceptible NRRL5267 strain resulting in a resistant strain could explain the loss of PsV-F in the group B mutants. As is the case with the *Saccharomyces cerevisiae* system discussed, resistance to one mycovirus does not necessarily carry over to other mycoviruses; thus the PsV-S replication continues in the group B mutants of *P. stoloniferum*. Although all group B mutants reported were tan in colour, this was not a requirement for loss of PsV-F since we have isolated green-spored strains derived from 5267 that contain only PsV-S.

The two reports of virus transmission in *Penicillium stoloniferum* partially support the theory of host resistance to PsV-F replication (Lhoas, 1971*a*, *b*) since when protoplasts of a virus-free *P. stoloniferum* strain were mixed with both PsV-F and PsV-S only the PsV-S could be detected in the mycelia derived from protoplasts. Transfer of PsV-F and PsV-S has also been reported by Lhoas (1971*b*) by heterokaryon formation between compatible strains of *P. stoloniferum*. However, the PsV-F level in the newly infected strain was much lower than the PsV-F level in the donor strain.

The specific mechanism of host resistance to mycovirus replication does not appear to be mycophenolic acid since this substance was not produced by the group B mutants. Fungal-mycoviral systems appear to be ideal for studying host–virus interactions in eucaryotic hosts. This system is advantageous because it is amenable to genetic alterations and to the isolation of gene products that may influence virus replication in the fungal host.

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